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Short communication

# Preparative isolation and purification of two isoflavones from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography

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**Abstract**

Two isoflavones, calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside, were separated from *n*-butanol extract of the root of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography in two steps using two different solvent systems composed of ethyl acetate–ethanol–*n*-butanol–water (30:10:6:50, v/v) and ethyl acetate–ethanol–water (5:1:5, v/v). From 200 mg of crude extract, calycosin-7-*O*-β-D-glycoside (12 mg) and formononetin-7-*O*-β-D-glycoside (10 mg) were isolated at over 95% purity by HPLC analyses, and their structures were identified by MS, $^{1}$H NMR and $^{13}$C NMR.


*Keywords:* *Astragalus membranaceus*; Counter-current chromatography; Plant materials; Preparative chromatography; Isoflavones; Calycosin glycoside; Formononetin glycoside; Glycosides

## 1. Introduction

The roots of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao, and certain species of *Astragalus* (Leguminosae) have long been used as an anti-perspirant, a diuretic or a tonic in Chinese traditional medicine under the name of Huang-qi in China and Ougi in Japan [1–4] while the studies on pharmacology and clinical practice have demonstrated the immunostimulating, cardiotonic and antiaging activities. Its isoflavones, including calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside, showed antimicrobial and superoxide anion scavenging activities [5–7].

The flavonoids were well known as one group of the beneficial components [2–4,8–10], and have suitable chromophores for UV detection so that it has been chosen as ''marker compounds'' for the chemical evaluation or standardization of Huang-qi and its products [4,11,12].

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The preparative separation and purification of calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside from plant materials by conventional methods is tedious and usually requires multiple chromatography steps. High-speed counter-current chromatography (HSCCC), being a unique liquid–liquid partition chromatography which uses no solid matrix, eliminates irreversible adsorption of sample onto the solid support [13] and therefore is considered as a suitable alternative for the separation of phenolic compounds such as flavonoids and hydroxyanthraquinones [14–16].

The present paper introduces a method for the separation of calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside, whose chemical structures are given in Fig. 1A, from a crude extract of *A. membranaceus* by HSCCC.

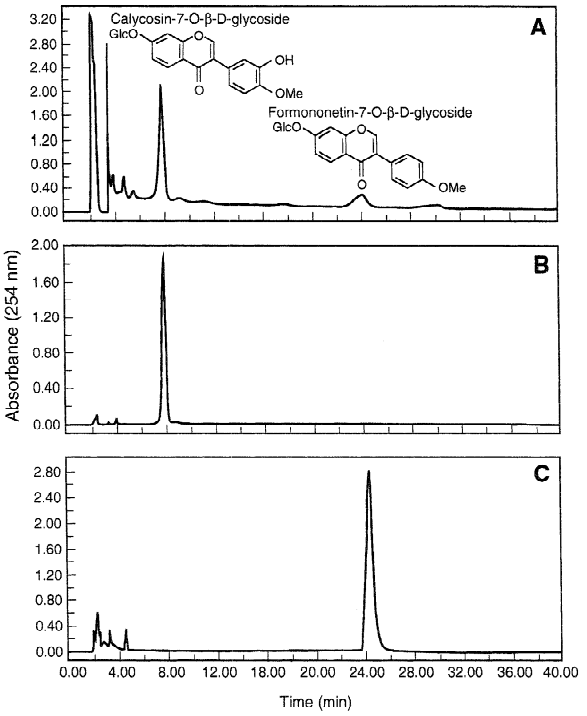


Fig. 1. The result of HPLC analyses of the crude ethyl acetate extract from *A. membranaceus* and repurified CCC fractions shown in Fig. 2B. (A) Crude sample; (B) peak 1 in Fig. 2B; (C) peak 2 in Fig. 2B. Column: SymmetryShield column (250 mm × 4.6 mm I.D.); column temperature: 35 °C; mobile phase: acetonitrile–water (20:80, v/v); flow-rate: 1.0 ml/min.

## 2. Experimental

### 2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS20 analytical high-speed counter-current chromatograph designed and constructed in Beijing Institute of New Technology Application (Beijing, China). The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 ml. The $\beta$ value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where $r$ is the distance from the coil to the holder shaft, and $R$, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 to 2000 rpm, an optimum speed of 1800 rpm was used in the present studies.

Preparative HSCCC was carried out using a Model GS10A2 multilayer coil of 1.6 mm I.D. and 110 m in length with a total capacity of 230 ml. The $\beta$ values of this preparative column range from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A manual sample injection valve with a 1.0-ml loop (for the analytical HSCCC) or a 20-ml loop (for the preparative HSCCC) (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column, respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Waters Millennium[32] system including a Waters 2487 dual-wavelength absorbance detector, a Waters 600E Multisolvent Delivery System, a Waters 600 System controller, a

Waters Delta 600 pump, and a Millennium[32] workstation (Waters, Milford, USA).

### 2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory, Tianjin, China.

### 2.3. Extraction of crude isoflavones

Raw roots of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao (47.5 kg) were extracted three times with 95% ethanol. Then the extract was combined and evaporated to dryness under reduced pressure, which yield 6.8 kg of dry powder. About 1 kg of the residue obtained from the combined extract was dissolved with 6 l of water. After filtration, the aqueous solution was extracted three times with 5 l each of water-saturated light petroleum (b.p. 60–90 °C), ethyl acetate and *n*-butanol successively which yielded 1 g of light petroleum extract, 25 g of ethyl acetate extract and 119 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. Then the *n*-butanol extract was chromatographed on polystyrene resin (NKA-0, 0.3–1.25 mm: NanKai Chemical Factory, Tianjin, China) with 40% ethanol. Portions of the above 40% ethanol extract were subjected to HSCCC.

### 2.4. Preparation of two-phase solvent system and sample solutions

For the present study, we selected two systems composed of ethyl acetate–ethanol–*n*-butanol–water (30:10:6:50, v/v) and ethyl acetate–ethanol–water (5:1:5, v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at suitable concentrations according to the analytical or the preparative purpose.

### 2.5. Separation procedure

For each separation, the analytical HSCCC was carried out with a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column inlet at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg in 1 ml of lower aqueous phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram. Preparative HSCCC was similarly carried out with a Model GS 10A2 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase as stationary phase. Then the sample solution (200 mg in 20 ml of lower aqueous phase) was injected through the sample port and the aqueous mobile phase was pumped through the column at a flow-rate of 2.0 ml/min while the column was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

### 2.6. HPLC analysis and identification of CCC peak fractions

The crude extract of *A. mongholicus* and each peak fraction from HSCCC were analyzed by HPLC. The analyses were carried out with a SymmetryShield column (250×4.6 mm I.D.) at a column temperature of 35 °C. The mobile phase composed of acetonitrile–water (20:80, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent monitored by a Waters 2487 dual-wavelength absorbance detector.

Identification of HSCCC peak fraction was carried out by MS, $^{1}$H NMR and $^{13}$C NMR spectra.

## 3. Results and discussion

The crude extract of *A. membranaceus* was first

analyzed by HPLC. The result indicates that the crude sample contains several compounds including calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside and some unknown compounds (Fig. 1A).

HSCCC separation was carried out using two different solvent systems. In the first separation, we examined the solvent systems composed of ethyl acetate–ethanol–*n*-butanol–water at different volume ratios such as 50:10:0:50, 50:5:5:50, 30:10:5:50, 25:15:5:50 and 30:10:6:50 using analytical HSCCC. The result indicated that the solvent system at a volume ratio of 30:10:6:50 was most suitable. For the second separation, the solvent systems composed of ethyl acetate–ethanol–water, ethyl acetate–methanol–water were examined at various volume ratios among which a solvent system composed of ethyl acetate–ethanol–water at the volume ratio of 5:1:5 could separate these two isoflavones well.

The sample was separated by HSCCC using the above two-phase solvent system composed of ethyl acetate–*n*-butanol–water (30:10:6:50, v/v) (Fig. 2A). After this separation, the peak fraction (shaded) containing calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside was collected and evaporated to dryness under reduced pressure. This fraction was further subjected to HSCCC using the second solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v). In the second separation shown in Fig. 2B, peaks 1 and 2 were identified as calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside, respectively, each with high purity of over 95% by HPLC analysis (Fig. 1B,C).

The two-step HSCCC separation described above yielded 12 mg of calycosin-7-*O*-β-D-glycoside and 10 mg of formononetin-7-*O*-β-D-glycoside from 200 mg of the crude extract.

The structural identification of the formononetin-7-*O*-β-D-glycoside was carried out by MS, $^{1}$H NMR and $^{13}$C NMR spectra. The MS data are as follows: Calycosin-7-*O*-β-D-glycoside: P-FAB-MS *m/z*: 447(M++1), 285(M++1-glucose unit); formononetin-7-*O*-β-D-glycoside: P-FAB-MS *m/z*: 431(M++1), 269(M++1-glucose unit). In NMR, $^{1}$H NMR (300 MHz, $C^{2}H_{3}O^{2}H$) $\delta$ ppm: 3.15–3.73 (6H), 3.78 (3H, s, OMe), 5.11 (1H, $J$=7.2 Hz, anomeric), 7.00 (2H, d, $J$=8.4 Hz, H-3′, H-5′), 7.15 (1H, dd, $J$=8.8, 2.2 Hz, H-6), 7.23 (1H, d, $J$=2.2

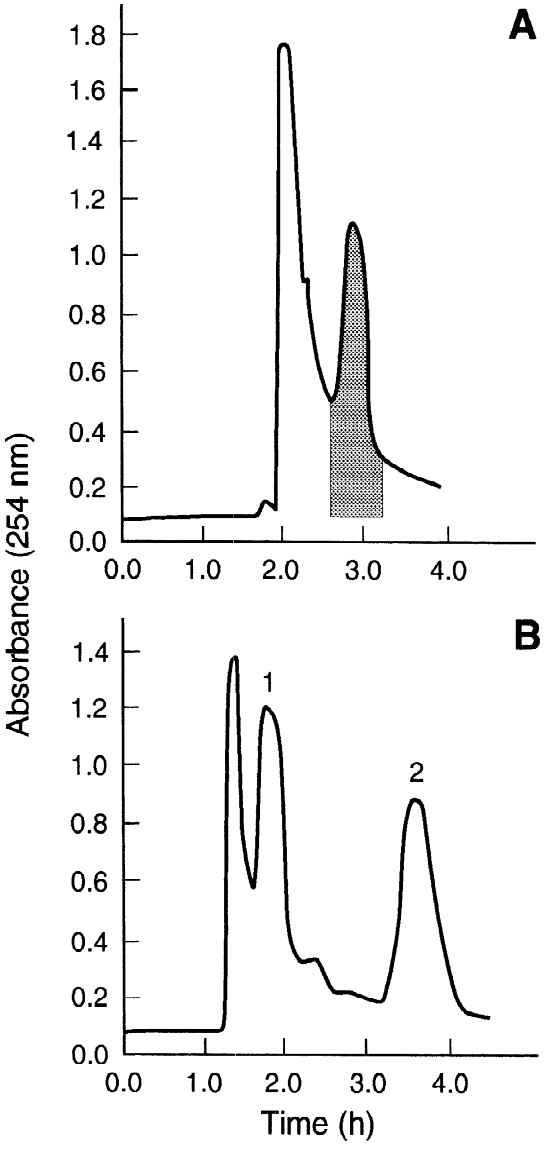


Fig. 2. HSCCC separation of extract from *A. membranaceus*. (A) Chromatogram of the crude ethyl acetate extract by preparative HSCCC. Solvent system: ethyl acetate–*n*-butanol–ethanol–water (30:10:6:50, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 200 mg dissolved in 20 ml lower phase; retention of the stationary phase: 60.0%. (B) Chromatogram of partially purified extract by HSCCC (shaded peak fraction in (A)). Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 80 mg dissolved in 20 ml lower phase; retention of the stationary phase: 66.7%.

Hz, H-8), 7.52 (2H, d, $J=8.4$ Hz, H-2′, H-6′), 8.04 (1H, d, $J=8.8$ Hz, H-5), 8.43 (1H, s, H-2). $^{13}$C NMR (300 MHz, $C^2H_3O^2H$) $\delta$ ppm: 174.7 (C-4), 161.4 (C-7), 159.0 (C-4), 157.1 (C-9), 153.7 (C-2), 130.1 (C-2 C-6), 127.0 (C-5), 124.0 (C-1), 123.4 (C-3), 118.4 (C-10), 115.6 (C-6), 113.6 (C-3 C-5), 103.4 (C-8), 100.0 (C-1), 77.2 (C-5), 76.5 (C-3), 73.1 (C-2), 69.6 (C-4), 60.6 (C-6), 55.1 (OMe).

The structural identification of the calycosin-7-*O*-β-D-glycoside was similarly carried out as follows: $^1$H NMR (300 MHz, $C^2H_3O^2H$) $\delta$ ppm: 3.25–3.73 (6H), 3.88 (3H, s, OMe), 5.11 (1H, $J=7.2$ Hz, anomeric), 6.97 (2H, brs, H-5′, H-6′), 7.05 (1H, s, H-2′), 7.19 (1H, dd, $J=8.7$, 2.1 Hz, H-6), 7.52 (1H, d, $J=8.4$ Hz, H-8), 8.13 (1H, d, $J=8.7$ Hz, H-5), 8.20 (1H, s, H-2). $^{13}$C NMR (300 MHz, $C^2H_3O^2H$) $\delta$ ppm: 177.9 (C-4), 163.5 (C-7), 159.2 (C-9), 155.3 (C-2), 149.2 (C-4′), 147.5 (C-3′), 128.3 (C-5), 126.0 (C-1′), 121.5 (C-6′), 120.2 (C-10), 117.3 (C-6), 117.0 (C-2′), 112.6 (C-5′), 104.9 (C-8), 101.8 (C-1″), 78.4 (C-5″), 77.8 (C-3″), 74.7 (C-2″), 71.2 (C-4″), 62.4 (C-6″), 56.4 (OMe).

The result of our studies clearly demonstrated that HSCCC is very useful in the preparative separation of calycosin-7-*O*-β-D-glycoside and formononetin-7-*O*-β-D-glycoside from the root extract of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao.

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